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Foetal development of skeletal muscle in bovines as a function of maternal nutrition, foetal sex and gestational age

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Summary

To determine the effects of maternal nutrition on modifications of foetal development of the skeletal muscle and possible increase in the potential of skeletal muscle growth in cattle, gestating cows were either fed 190% NRC recommendations (overnourished; ON) or 100% NRC recommendation (control; CO). Interaction between maternal nutrition (MN) and the foetal sex (FS) was also investigated. Foetuses were necropsied at four different time points throughout gestation (139, 199, 241 and 268 days of gestation) to assess the mRNA expression of myogenic, adipogenic and fibrogenic markers in skeletal muscle. Phenotypic indicators of the development of skeletal muscle fibres, intramuscular lipogenesis and collagen development were also evaluated. Modifications in mRNA expression of skeletal muscle of foetuses were observed in function of MN and FS despite the lack of effect of MN and FS on foetal weight at necropsy. Maternal ON increased the mRNA expression of the myogenic marker *Cadherin-associated protein, beta 1* (*CTNNB1*) and adipogenic markers *Peroxissome proliferator-activated receptor gamma* (*PPARG*) and *Zinc finger protein 423* (*ZNF423*) at midgestation. However, no differences on foetal skeletal muscle development were observed between treatments at late gestation indicating that a compensatory development may have occurred on CO foetuses making the effect of MN on skeletal muscle development not significant at late gestation. Moreover, our data have shown an evidence of sexual dimorphism during foetal stage with a greater skeletal muscle development in male than in female foetuses. In conclusion, providing a higher nutritional level to pregnant cows changes the trajectory of the development of skeletal muscle during midgestation, but apparently does not change the potential of post-natal growth of muscle mass of the offspring, as no differences in skeletal muscle development were observed in late gestation.

KEYWORDS

adipogenesis, fibrogenesis, foetal programming, myogenesis, sexual dimorphism, zebu

1 | INTRODUCTION

Great efforts have been made to support the intensification and possible benefits of pasture-based cattle ranching by demonstration of increased productivity and efficiency of use of pasture when grazed by growing animals (Bouman & Nieuwenhuyse, 1999; Detmann, Gionbelli, & Huhtanen, 2014; Detmann, Paulino, & Valadares Filho, 2010; Sampaio et al., 2010; Valente et al., 2012, 2013). Although

breeding herd represents approximately 42% of the total herd, the impacts of nutrition on this group of animals have not received a deserved interest along the last decades (Gionbelli et al., 2015). The nutritional negligence is commonly seen in tropical regions such as Brazil where beef cattle production is based on pastures and the breeding herd faces the effects of distribution and seasonal variation in quantity and quality of forage along the year. Moreover, in grazing systems, the rainy season promotes a better availability of forage and aligns with the breeding season in the tropics; therefore pregnant cows usually experience feed restriction during mid to late gestation, which overlaps with the dry season in most of the tropical cattle production areas (Paulino & Duarte, 2014).

Such scenario raises questions and need of more foetal programming studies in beef systems as a model to better understand how manipulation of the intra-uterine development minimises environmental effects on the offspring performance throughout their post-natal life. Therefore, considerably research has shown that maternal nutrition during pregnancy alters intestinal (Duarte et al., 2013; Hammer et al., 2011; Meyer et al., 2010; Prezotto et al., 2016; Reed et al., 2007; Trahair, DeBarro, Robinson, & Owens, 1997; Yanusova et al., 2013) and muscular (Du et al., 2010; Duarte et al., 2014; Vonnahme, 2007; Wu, Bazer, Wallace, & Spencer, 2006) development of the ruminants' foetuses with effects in muscle and internal organs development that can be permanent (Underwood et al., 2010; Wu et al., 2006). Moreover, it has also been suggested that maternal nutrition may program the mesenchymal stem cells in the skeletal muscle to undergo adipo/fibrogenic differentiation, which predisposes the offspring to intramuscular fat deposition later in life (Du et al., 2013).

Herein, we investigated the effects of overnourishing (ON) gestating cows on the development of skeletal muscle of the foetus. Additionally, we have also investigated the effects of foetal sex (FS) and the interactions with maternal nutrition (MN) and days of gestation (DG) on the development of skeletal muscle cells.

2 | MATERIALS AND METHODS

2.1 | Animal management and experimental diets

All animal care and handling procedures were approved by Animal Care and Use Committee of the Department of Animal Science of the *Universidade Federal de Viçosa*, *Viçosa*, *Minas Gerais*, Brazil, prior to initiation of the experiment. Forty-four multiparous non-lactating Holstein × Gyr cows with initial body weight and age averaging 480 ± 10.1 kg and 5 ± 0.5 years old, respectively, were confirmed pregnant from a single bull at day 30 of gestation via ultrasonography after a fixed-time artificial insemination protocol. The general procedures for the synchronisation and artificial insemination protocol as well the nutritional management of the cows during the adaptation period have been previously described by Rotta et al. (2015). Day of insemination was considered day 0. At 60 days of gestation foetal sexing was performed by real-time ultrasonography and animals were randomly allocated to one of the two feeding treatments which lasted until the slaughter of each group. Cows were randomly divided into two treatment groups: (i) control group (CO, n = 24), which cows were fed 1.15% of body weight per day calculated to maintain body weight throughout gestation; (ii) overnourished (ON, n = 20), which cows were fed ad libitum with the same diet composition as the CO group. The diet was based on corn silage (93%), cotton meal (5%) and mineral + urea mixture (2%). More details on the diet are provided by Rotta et al. (2015). Both groups were fed twice daily (60% in the morning and 40% afternoon).

2.2 | Skeletal muscle tissue sampling

To evaluate the effects of maternal nutrition and progression of gestation on foetal skeletal muscle development at different stages of gestation, cows and its foetuses were necropsied at 139 (n = 9), 199 (n = 11), 241 (n = 11) and 268 days (n = 10) of gestation, as a representation of 50%, 70%, 85% and 95% of gestation length (Mellado, Coronel, Estrada, & Ríos, 2011) and 18%, 31%, 73% and 96% of total growth of the gravid uterus (Gionbelli et al., 2015). Pre-harvest animal care and handling procedures followed the National Sanitary and Industrial Inspection Regulation for Animal Origin Products (Brasil 1997). Briefly, feed was withdrawn from animals 16 hr prior to harvesting; however, ad libitum access to water was allowed. Euthanasia was performed using captive bolt stunning and exsanguination. The euthanasia of foetuses was performed in accordance with the American Veterinary Medical Association Guidelines (AVMA 2013). After exsanguination was performed in the cow, the gravid uterus was collected and foetuses were removed. Samples of *Longissimus dorsi* muscle (LM) from the foetuses were collected from both sides of the carcass after the removal of skin and apparent subcutaneous fat. A total of three samples were collected from each foetus. One of the three LM samples was placed in sterile tubes containing RNAlater (Qiagen, Hilden, North Rhine, Westphalia, Germany), stored at 4°C overnight, and then kept at −80°C until RNA isolation was performed. Another sample was fixed in fresh 10% (wt/vol) formalin in phosphate buffer (pH = 7.4) immediately after slaughter and then processed for histological analysis. The third sample was fresh frozen at −20°C for further ether extract and crude protein quantification.

2.3 | mRNA extraction and mRNA expression analysis

Total RNA (1 μg) was extracted from 0.5 g of powdered tissue samples using TRIzol reagent (Invitrogen, Carlsbad, California, USA), treated with DNase I, Amplification Grade (Invitrogen), and reverse transcribed into cDNA using the GoScript Reverse Transcription System (Promega, Madison, Wisconsin, USA). The primer sets used are shown in Table 1. Reverse transcription PCR was performed on a 7300 Real-Time PCR System (Applied Biosystems, Foster City, California, USA) using SYBR Green RT-PCR kit from Bio-Rad and the following cycle parameters: 95°C for 3 min and 40 cycles at 95°C for 10 s and 60°C for 30 s. The amplification efficiency was 0.90 to 0.99. After amplification, a melting curve (0.01°C/s) was used to confirm product purity. Results are expressed relative to RPS18 using the $2^{\Delta\Delta Ct}$ method

TABLE 1 Primers for genes analyzed by qRT-PCR

Gene	Gene abbreviation	UniGene access code	Forward sequence	Reverse sequence
Collagen type I, alpha 1	*COL1A1*	NM_001034039.1	CCACCCCAGCCGCAAAGAGT	ACGCAGGTGACTGGTGGGATGTC
Collagen type III, alpha 1	*COL3A1*	NM_001076831.1	GGCCCCCTGGAAAGGACGGA	CCCCGCCAGCACCACAACAT
Fibronectin 1	*FN1*	NM_001163778.1	GCGTGTCACCTGGGCTCCAC	CGGTGCCGGGCAGGAGATTT
Transforming growth factor, beta 1	*TGFB1*	NM_001166068.1	AGCCAGGGGGATGTGCCA	TAGCACGCGGGTGACCTCCT
CCAAT enhancer-binding protein, alpha	*CEBPA*	NM_176784.2	TGCGCAAGAGCCGGGACAAG	ACCAGGGAGCTCTCGGGCAG
Peroxissome proliferator-activated receptor gamma	*PPARG*	NM_001098905.1	TGGAGACCGCCCAGGTTTGC	AGCTGGGAGGACTCGGGGTG
Zinc finger protein 423	*ZNF423*	NM_001101893.1	GGATTCCTCCGTGACAGCA	TCGTCCTCATTCCTCTCCTCT
Cadherin-associated protein, beta 1	*CTNNB1*	NM_001076141.1	CCGGCTATTGTAGAAGCTGGTG	AAGGCGCTGACTTGGATCTGTC
Myogenic differentiation 1	*MYOD1*	NM_001040478.2	TTCCGACGGCATGATGGACTAC	TAAGTGCGGTCGTAGCAGTTCC
Myogenin (Myogenic factor 4)	*MYOG*	NM_001111325.1	TACAGACGCCCACAATCTGCAC	AGCGACATCCTCCACTGTGATG
Ribosomal protein S18	*RPS18*	NM_001033614	CCTGCGGCTTAATTTGACTC	AACTAAGAACGGCCATGCAC

(Livak & Schmittgen, 2001). The stability expression of RPS18 among experimental treatments was determined through statistical analysis by Tukey's method at α=0.05 to use RPS18 as a reference gene. No variation of RPS18 expression was observed (p = .845) among experimental treatments.

2.4 | Skeletal muscle histochemical analysis

To determine the number of myocytes, fixed skeletal muscle samples were embedded in resin using the HistoResin Mounting Kit (Leica, Solmos, Hessen, Germany). Sections were cut at 3 μm using a RM2255 microtome (Leica Microsystems Inc., Wetzlar, Germany), stained with toluidine blue, mounted in synthetic resin, and analyzed under an EVOS xl light microscope (AMG, Bothell, Washington, USA) for observation of number of muscle cells. A total of 10 images per foetus were taken (two images per section and five sections per foetus) at 400 × magnification to quantify a total of muscle cells using ImageJ software (National Institute of Health, Baltimore, Maryland, USA). To quantify the amount of intramuscular collagen, fixed skeletal muscle samples were embedded in Paraplast (Sigma-Aldrich, St. Louis, Missouri, USA) and coronal sections (5 μm) were cut using a RM2245 microtome (Leica Microsystems Inc.). Sections were deparaffinised through incubations on HistoChoice (Sigma-Aldrich) and then rehydrated through incubations on ethanol solutions. After rehydration, sections were stained for 1 hr in the picrosirius solution (Junqueira, Bignolas, & Brentani, 1979). Picrosirius solution was composed of 0.1% (wt/vol) of Direct Red 80 (Sigma-Aldrich) in 1.3% (wt/vol) aqueous picric acid solution. After one hour incubation, sections were washed for 2 min in 0.01 *N* HCl solution, dehydrated, cleared and mounted on synthetic resin. Picrosirius-stained sections were observed under polarised light using an Olympus BX53 coupled with a DP21 digital camera (Olympus, Tokyo, Japan). For quantification of picrosirius-stained intramuscular collagen, a total of 10 images per foetus (two images per section and five sections per foetus) were taken using the same background under the polarised light and then analyzed using ImageJ software. All the images were converted into grayscale and split into red, green and blue channels. The green channel of all images was thresholded to the same level to highlight the stained collagen. Collagen was quantified as percentage of the total image area. All images were analyzed for collagen quantification at 100 × magnification.

2.5 | Chemical analysis

Ether extract quantification and crude protein content in the foetal skeletal muscle follow the procedures described by Lage et al. (2012) for adult animals.

2.6 | Statistical analysis

The model utilised for data analyzed included MN, foetal sex and DG (139,199, 241 and 268 days) as fixed effects, and their interactions as follows:

$$Yijkl = \mu + MN_i + FS_j + DG_k + (MN \times FS)_{ij} + (MN \times DG)_{ik} + (FS \times DG)_{jk} + (MN \times FS \times DG)_{ijk} + e_{ijkl}$$

where MN_i is the *i*th level of the fixed effect of maternal nutrition, FS_j is the *j*th level of the fixed effect foetal sex, DG_k is the *k*th level of the fixed effect days of gestation and e_{ijkl} is the random error associated with Y_{ijkl}. Gene expression levels were transformed using the natural logarithm of the expression values + 1 to achieve normality (Voge et al., 2004). Outliers were removed to achieve normality using Shapiro–Wilks test at $\alpha = 0.10$ (Shapiro & Wilk, 1965). Least square means were estimated for all effects and compared using Tukey's method. Significance was declared at $p \leq .05$ and a tendency was reported if $.05 < p < .10$. All statistical procedures were performed using the MIXED procedure of SAS (SAS, version 9.2, software; SAS Institute).

3 | RESULTS

3.1 | Maternal and foetal body weight

From the forty-four cows used in this experiment, one had twin pregnancy and two aborted. Thus, the data presented are from the results obtained from the 41 singleton pregnancies. Overnourished cows were heavier ($p < .001$) than CO cows at slaughter (Table 2), showing the efficacy of our maternal nutrition treatments. Average daily dry matter intake (DMI) was 6.38 ± 0.32 (mean ± *SE*) and 11.24 ± 0.37 kg/day for CO and ON-fed cows respectively. Cow BW at each period of slaughter was not affected by FS ($p = .976$). Interactions between nutritional treatment, FS and DG were not observed ($p \geq .557$). Foetal body weight (Table 2) was affected by DG ($p < .001$) but not by FS. Thus, it can be noted that the effects of MN, FS and DG on muscular development of foetuses occurred independently of the foetal size.

3.2 | mRNA expression of myogenic markers and phenotypic indicators of myogenesis

The mRNA expression for *Cadherin-associated protein, β1* (*CTNNB1*) was affected by all fixed effects evaluated ($p \leq .03$; Table 3). Interaction between MN and DG showed that *CTNNB1* mRNA expression was greater in ON foetuses only at 139 days of pregnancy ($p < .05$) while similar values for its mRNA expression was observed between foetuses from both MN groups in the subsequent stages of pregnancy ($p > .05$). Similar values of mRNA expression for *Myogenic differentiation I* (*MYOD1*; $p = .51$) and *Myogenin* (*MYOG*; $p = .32$) were observed among ON and CO foetal muscle (Table 3). The mRNA expression of *MYOD1* was 16% greater in skeletal

TABLE 2 Least square means ± standard errors of the means of the effects of maternal feeding level, foetal sex and days of gestation on initial and final cow live weight and foetal weight

Item	Maternal nutrition[1]		Foetal sex		Days of gestation				*p*-value[2]						
	CO (*n* = 23)	ON (*n* = 18)	Female (*n* = 21)	Male(*n* = 20)	139 (*n* = 9)	199 (*n* = 11)	241 (*n* = 11)	268 (*n* = 10)	*N*	S	D	*N*×S	*N*×D	S×D	N×S×D
Cows initial weight, kg	477 ± 11	477 ± 12	476 ± 12	479 ± 12	489 ± 19	478 ± 15	482 ± 15	460 ± 18	.992	.860	.688	.745	.661	.839	.600
Cows final weight, kg	523 ± 14	646 ± 15	584 ± 14	585 ± 15	558 ± 23	574 ± 19	596 ± 19	611 ± 22	<.001	.976	.344	.646	.557	.578	.694
Foetal weight at slaughter, kg	16.4 ± 0.7	17.3 ± 0.7	17.1 ± 0.7	16.5 ± 0.7	1.84[d]±1.06	10.0[c]±0.8	22.8[b]±0.9	32.8[a]±1.0	.330	.518	<.001	.925	.885	.367	.893

[1]CO, control and ON, overnourished cows.
[2]The main effects of maternal nutrition (*N*), foetal sex (S) and days of gestation (D).
[a-d]Within a variable, means differ ($p < .05$).

TABLE 3 Least square means ± standard errors for mRNA expression of myogenic markers and phenotypic indicators of myogenesis evaluated on foetal skeletal muscle of Holstein × Gyr cattle according to maternal nutrition, foetal sex and days of gestation

	Maternal nutrition[1]		Foetal sex		Days of gestation				*p*-value[2]						
Item	CO (*n* = 23)	ON (*n* = 18)	Female (*n* = 21)	Male (*n* = 20)	139 (*n* = 9)	199 (*n* = 11)	241 (*n* = 11)	268 (*n* = 10)	*N*	S	D	*N*×S	*N*×D	S×D	*N*×S×D
mRNA expression of myogenic markers (arbitrary units)															
CTNNB1	2.25 ± 0.08	2.50 ± 0.08	2.19 ± 0.08	2.56 ± 0.08	2.78 ± 0.13	2.63 ± 0.11	2.16 ± 0.11	1.93 ± 0.11	.035	.003	.002	.986	<.001	.054	.140
CO	-	-	-	-	2.10 ^{cd}±0.20	2.57^{bc}±0.14	2.22 ^{cd}±0.14	2.10^{ef}±0.14	-	-	-	-	-	-	-
ON	-	-	-	-	3.45^{a}±0.18	2.69^{ab}±0.16	2.09^{de}±0.16	1.76^{f}±0.17	-	-	-	-	-	-	-
MYOD1	2.61 ± 0.11	2.72 ± 0.13	2.47 ± 0.12	2.87 ± 0.13	3.31^{a}±0.20	3.09^{a}±0.15	2.27^{b}±0.16	2.01^{c}±0.17	.508	.033	<.001	.856	.122	.288	.444
MYOG	3.74 ± 0.11	3.92 ± 0.12	3.70 ± 0.11	3.97 ± 0.13	4.32^{a}±0.19	4.67^{a}±0.14	3.63^{b}±0.14	2.72^{b}±0.19	.320	.120	<.001	.486	.250	.254	.364
Phenotypic indicators of myogenesis															
Crude protein content of skeletal muscle (g/kg)	117 ± 2	122 ± 2	120 ± 2	119 ± 2	82.9^{d}±3.6	112^{c}±2	135^{b}±2	148^{a}±2	.051	.849	<.001	.885	.458	.618	.150
Number of myocytes (400-fold magnification)	379 ± 9	382 ± 9	362 ± 9	399 ± 9	459^{a}±15	458^{a}±12	324^{b}±12	280^{c}±14	.796	.009	<.001	.555	.091	.291	.247

[1]CO, control and ON, overnourished cows.
[2]The main effects of maternal nutrition (*N*), foetal sex (S) and days of gestation (D).
[a-f]Within a variable, means differ ($p < .05$).

muscle from males than from females (p = .03). However, mRNA expression of *MYOG* expression was not affected by foetal sex (p = .12). No interactions were observed among the fixed effects evaluated on *MYOD1* ($p \geq .12$) and *MYOG* ($p \geq .25$) mRNA expression. The three myogenic markers evaluated were less expressed in foetal muscle at late gestation when compared to midgestation ($p \leq .002$, Table 3).

The content of crude protein (CP) in foetal muscle (g/kg) tended to be greater (p = .05) in foetuses from ON cows compared to those from CO cows. Moreover, CP was increased ($p < .001$) in both groups as gestation progressed (Table 3), being 78% greater at 268 days of gestation compared to 139 days. Finally, foetal sex had no effect (p = .85) on CP content of foetal skeletal muscle.

The number of muscle cells was 10% greater (p = .009) in males compared to females foetuses (Table 3). Maternal nutrition did not affect (p = .80) the number of myocytes. A greater number of myocytes in LM of bovine foetuses was observed during midgestation when compared to late gestation ($p < .001$).

3.3 | mRNA expression of adipogenic markers and intramuscular fat content

The mRNA expression for the early adipogenic marker *zinc finger protein 423* (*ZNF423*) was affected by interactions between the main effects evaluated. Interaction between MN and DG (p = .001) has shown that *ZNF423* was expressed greater in skeletal muscle of foetuses from ON-fed cows than in those from CO-fed cows at 139 days of gestation ($p < .05$). No differences in *ZNF423* expression were observed during the subsequent stages of gestation ($p > .05$). Similarly, a greater mRNA expression of *ZNF423* was observed in skeletal muscle of males than in females at 139 days of gestation ($p < .10$), but not in other gestational periods evaluated ($p > .10$). Although interactions were observed, no differences among MN levels and foetal sex were detected at 268 days of gestation in mRNA expression of *ZNF423*. The mRNA expression of *CCAAT enhancer-binding protein alpha* (*CEBPA*) was not affected by interactions between the main effects evaluated ($p \geq .21$, Table 4) showing similar values in skeletal muscle of CO and ON foetuses (p = .79). However, *CEBPA* expression was greater in male than in female foetuses (p = .03) and lower at late gestation compared to midgestation ($p < .001$, Table 4). The mRNA expression of *Peroxisome proliferator-activated receptor gamma* (*PPARG*) was 26% greater in male than in female foetuses (p = .002, Table 4). A greater mRNA expression of *PPARG* was also observed in skeletal muscle from ON foetuses than in CO foetuses at 139 days of gestation ($p < .05$); however, no differences were observed among MN groups at the subsequent stages of gestation ($p > .05$).

Although some effects of MN were observed on mRNA expression of adipogenic markers, the intramuscular fat content was not affected by MN (p = .89). Male and female foetuses also presented similar contents of intramuscular fat during pregnancy (p = .19). Foetal muscle fat content increased by approximately 36% from 139 days of gestation to the subsequent stages (p = .02, Table 4).

3.4 | mRNA expression of fibrogenic markers and phenotypic quantification of intramuscular collagen deposition

The mRNA expression of *Collagen type I alpha 1* (*COL1A1*) tended to be greater in ON than in CO foetuses (p = .091, Table 5) and in male than female foetuses (p = .095). The mRNA expression of *COL1A1* also decreased from 199 days and subsequent stages of gestation ($p < .001$, Table 5). An interaction between foetal sex and DG was observed for mRNA expression of *Fibronectin 1* (*FN1*, p = .002), when the mRNA abundance was greater in males than in females ($p < .05$) only at 139 days of gestation (Table 5), with no differences observed at the subsequent periods ($p > .05$). The mRNA expression of *FN1* was greater in ON than in CO foetuses (p = .03). The mRNA expression of *transforming growth factor* β (*TGFB 1*) was affected by days of gestation (p = .004), being greater at 199 and decreasing at 241 and 268 days of gestation.

To evaluate the phenotypic fibrogenesis quantification of collagen, deposition in foetal muscle was performed via a histochemical approach (Figure 1). The intramuscular collagen deposition was not affected by MN (p = .91) and FS (p = .23, Table 5). However, an increase in collagen deposition was observed in response to the progression of gestation ($p < .001$). Collagen content increased approximately five times from 139 to 268 days of gestation (Table 5).

4 | DISCUSSION

All effects on foetal skeletal muscle development observed in this study occurred without differences in foetal BW within the same gestational period. These findings are in agreement with previous reports which have shown that the vast majority of changes in the trajectory of foetal development in response to changes in the uterine environment occur without changing the birthweight (Du et al., 2010; Trahair et al., 1997; Underwood et al., 2010; Wu et al., 2006).

Both maternal nutrient restriction (Zhu et al., 2006) and obesity (Tong et al., 2009) have been shown to decrease myogenesis in ruminants. However, our results are not in agreement with the same noted above as we did not observe a decrease in myogenesis in foetal skeletal muscle in response to maternal ON. We observed an increase in expression of *CTNNB1* mRNA associated with an increased number of myocytes at 139 days of gestation in the group of maternal ON; although, no differences were observed among MN levels in the subsequent time points of gestation. In a previous study using beef cattle as a model, maternal overnutrition tended to decrease gene expression of *CTNNB1* (Duarte et al., 2014), contrary to what we observed in this study. Perhaps, this may suggest that maternal nutrition affects differently the foetal muscular development in breeds selected for beef production over cross-bred animals composed of dairy and beef breeds, as the animals used in this study was Holstein x Gyr cross-bred cows. The differences in expression of *CTNNB1* between ON and CO foetuses may be explained by a higher availability of nutrients to the gravid uterus of ON cows, which promotes increased myogenic

TABLE 4 Least square means ± standard errors for mRNA expression of adipogenic markers and muscle fat content as a phenotypic indicator of lipogenesis evaluated on foetal skeletal muscle of Holstein × Gyr cattle according to maternal nutrition, foetal sex and days of gestation

	Maternal nutrition[1]		Foetal sex		Days of gestation				*p*-value[2]						
Item	CO (*n* = 23)	ON (*n* = 18)	Female (*n* = 21)	Male (*n* = 20)	139 (*n* = 9)	199 (*n* = 11)	241 (*n* = 11)	268 (*n* = 10)	*N*	S	D	*N*×S	*N*×D	S×D	*N*×S×D
mRNA expression of adipogenic markers (arbitrary units)															
ZNF423	2.32 ± 0.10	2.62 ± 0.09	2.20 ± 0.09	2.74 ± 0.11	3.14 ± 0.15	3.02 ± 0.12	2.11 ± 0.14	1.61 ± 0.16	.049	<.001	<.001	.581	.001	<.001	.307
CO	-	-	-	-	2.43^{c} ± 0.21	3.08^{b} ± 0.16	2.15^{cd} ± 0.22	1.62^{de} ± 0.22	-	-	-	-	-	-	-
ON	-	-	-	-	3.86^{a} ± 0.19	2.95^{b} ± 0.17	2.05^{cde} ± 0.17	1.59^{e} ± 0.22	-	-	-	-	-	-	-
Female	-	-	-	-	2.26^{C} ± 0.23	2.86^{B} ± 0.15	2.08^{CD} ± 0.15	1.57^{E} ± 0.15	-	-	-	-	-	-	-
Male	-	-	-	-	4.03^{A} ± 0.17	3.17^{B} ± 0.17	2.12^{CD} ± 0.23	1.64^{DE} ± 0.27	-	-	-	-	-	-	-
CEBPA	2.93 ± 0.11	2.97 ± 0.11	2.77 ± 0.11	3.13 ± 0.11	3.02^{ab} ± 0.17	3.39^{a} ± 0.15	2.56^{c} ± 0.15	2.82^{cd} ± 0.15	.791	.029	<.001	.384	.377	.624	.209
PPARG	2.21 ± 0.11	2.45 ± 0.10	2.07 ± 0.11	2.60 ± 0.11	2.90 ± 0.16	2.82 ± 0.15	2.08 ± 0.16	1.52 ± 0.14	.128	.002	<.001	.482	.008	.190	.168
CO	-	-	-	-	2.32^{cd}±0.23	2.58^{bc}±0.21	2.27^{cd}±0.24	1.66^{ef}±0.19	-	-	-	-	-	-	-
ON	-	-	-	-	3.48^{a}±0.21	3.06^{ab}±0.21	1.90^{de}±0.21	1.38^{f}±0.21	-	-	-	-	-	-	-
Phenotypic indicator of lipogenesis															
Fat content of skeletal muscle (g/kg)	6.10 ± 0.26	6.05 ± 0.29	5.81 ± 0.26	6.34 ± 0.29	4.78^{b}±0.52	6.25^{a}±0.34	6.99^{a}±0.34	6.28^{a}±0.35	.891	.191	.017	.906	.887	.968	.565

[1]CO, control and ON, overnourished cows.
[2]The main effects of maternal nutrition (*N*), foetal sex (S) and days of gestation (D).
[a-f] Within a variable, means differ ($p < .05$).
[A-E] Within a variable, means differ ($p < .05$).

TABLE 5 Least square means ± standard errors for mRNA expression of fibrogenic markers and intramuscular collagen deposition as a phenotypic indicator of fibrogenesis evaluated on foetal skeletal muscle of Holstein × Gyr cattle according to maternal nutrition, foetal sex and days of gestation

	Maternal nutrition[1]		Foetal sex		Days of gestation				*p*-value[2]						
Item	CO (*n* = 23)	ON (*n* = 18)	Female (*n* = 21)	Male (*n* = 20)	139 (*n* = 9)	199 (*n* = 11)	241 (*n* = 11)	268 (*n* = 10)	*N*	S	D	*N*×S	*N*×D	S×D	*N*×S×D
mRNA expression of fibrogenic markers (arbitrary units)															
COL1A1	4.06 ± 0.20	4.56 ± 0.20	4.06 ± 0.20	4.56 ± 0.20	5.15^{a}±0.32	5.51^{a}±0.26	3.76^{b}±0.26	2.84^{c}±0.28	.091	.095	<.001	.953	.739	.130	.198
COL3A1	3.77 ± 0.15	4.05 ± 0.15	3.72 ± 0.15	4.11 ± 0.15	4.34 ± 0.25	4.95 ± 0.20	3.73 ± 0.20	2.62 ± 0.21	.216	.085	<.001	.382	.844	.072	.154
FN1	3.59 ± 0.12	4.02 ± 0.13	3.63 ± 0.12	3.98 ± 0.13	4.55 ± 0.20	4.90 ± 0.16	3.41 ± 0.17	2.36 ± 0.20	.027	.069	<.001	.806	.121	.002	.201
Female	-	-	-	-	3.62^{c}±0.32	4.93^{b}±0.21	3.43^{c}±0.21	2.55^{d}±0.21	-	-	-	-	-	-	-
Male	-	-	-	-	5.48^{a}±0.23	4.86^{b}±0.24	3.39^{c}±0.26	2.18^{d}±0.33	-	-	-	-	-	-	-
TGFB1	3.27 ± 0.13	3.22 ± 0.13	3.29 ± 0.11	3.21 ± 0.13	3.43^{ab}±0.18	3.69^{a}±0.18	3.18^{b}±0.16	2.67^{c}±0.18	.759	.629	.004	.100	.812	.624	.747
Phenotypic indicator of fibrogenesis															
Intramuscular collagen deposition (percentage of area at 100-fold magnification)	2.56 ± 0.09	2.55 ± 0.10	2.64 ± 0.09	2.47 ± 0.10	0.70^{c}±0.15	2.72^{b}±0.13	3.33^{a}±0.13	3.46^{a}±0.14	.907	.227	<.001	.974	.203	.656	.907

[1]CO, control and ON, overnourished cows.
[2]The main effects of maternal nutrition (*N*), foetal sex (S) and days of gestation (D).
[a-d]Within a variable, means differ ($p < .05$).

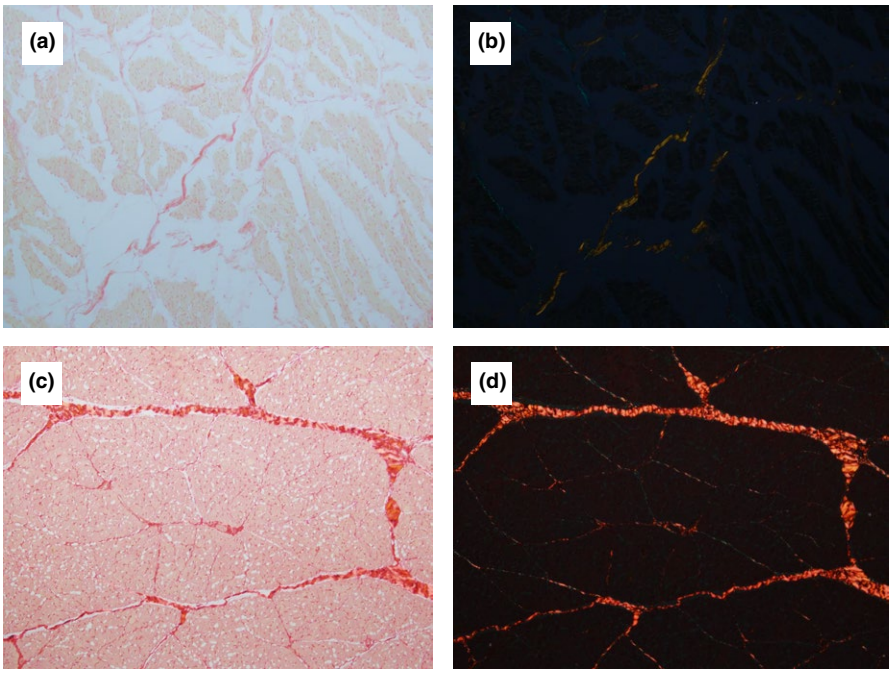

FIGURE 1 Influence of days of gestation on collagen area of Longissimus muscle (LM) of bovine foetuses. Representative images of LM showed in the figure were stained with picrosirius and observed under brightfield light (a and c) and polarised light (b and d) at 100-fold magnification. Images are from 139 (a and b) and 268 (c and d) days of gestation. Intramuscular collagen deposition (percentage of area at 100-fold magnification) in LM of bovine foetuses was not affected by maternal nutrition ($p = .907$) and foetal sex ($p = .227$); however, increased as gestation progressed ($p < .001$)

function in the developing foetus (Du et al., 2010). Nonetheless, myogenesis seemed to have occurred at a compensatory rate in foetuses from CO-fed cows and it may be occurred due to the fact that the CO group did not experience feed restriction throughout gestation. Evidence of compensatory foetal muscle growth from nutrient restricted and then re-fed cows has been recent reported (Gonzalez et al., 2013) and seems to be related to the effects that were observed in our study.

In general, the mRNA expression of all myogenic markers and the number of myocytes decreased from midgestation to late gestation. These findings are in agreement with those previously observed by Duarte et al. (2014) in beef cattle foetuses and revised by Du et al. (2010) using data from studies in sheep, rodents and humans. The observed decrease in myogenesis as gestation progresses up to midgestation and subsequent intensification after midgestation (4.5 mo), occurs due to the formation of new muscle cells being reduced as increase in intramuscular adipogenesis occurs (Du et al., 2010). Increase in fat content of the muscle was observed in this study from mid gestation to late gestation, indicating an increase in intramuscular adipogenesis in the last third of gestation. However, the mRNA expression of adipogenic markers was observed to decrease from midgestation to late gestation instead of an expected increase.

It was expected that ON treatment would promote increased adipogenesis in the foetal skeletal muscle based on previous reports (Duarte et al., 2014; Tong et al., 2009). An enhancement of mRNA expression of adipogenic markers was observed for two of the three markers evaluated at 139 days of gestation, with no effects on subsequent periods of gestation. The fat content of skeletal muscle of foetuses did not change as a function of MN in this study (Table 4).

Previous studies have suggested that the *CTNNB1* can alter the expression of other myogenic markers by regulating the expression of *paired box 3* (*PAX3*), which acts upstream of *MYOD1* during skeletal muscle development (Gustafsson et al., 2002; Ridgeway & Skerjanc, 2001). The *CTNNB1* can also downregulate myogenesis by regulating

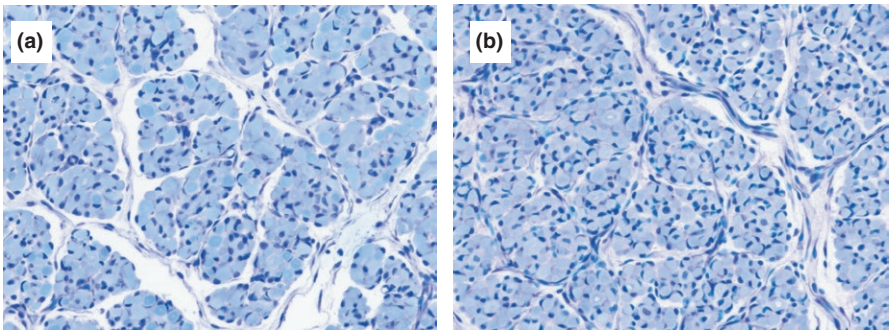

FIGURE 2 Influence of maternal nutrition (control [CO] and overnourishing [ON]) on number of myocytes of Longissimus muscle (LM) of bovine foetuses at 139 days of gestation. Representative images of foetal LM showed in this figure were stained with toluidine blue at 400-fold magnification from a CO (a) and ON (b) male foetuses. Number of myocytes tended to be greater in foetuses from ON-fed cows at midgestation ($p < .10$, 139 days of gestation, as exemplified in this figure) but did not differ at late gestation ($p > .05$)

the expression of *PPARG* (Okamura et al., 2009). Although in the current study the results of *CTNNB1* and *MYOD1* expression were correlated, there was no signal of *PPARG* downregulation by *CTNNB1*, with the pattern of the mRNA expression of both genes being similar (Tables 3 and 4). Our finding was different than previous reports when greater expression of *PPARG* was observed in response to decreased expression of *CTNNB1* (Duarte et al., 2014).

To our knowledge, this is the first study investigating the effects of gender on myogenesis, adipogenesis and fibrogenesis of skeletal muscle in bovines during the foetal phase. The muscle mRNA expression of myogenic markers *CTNNB1* and *MYOD1* were greater in male than in female foetuses, as well the expression of all adipogenic markers evaluated (*ZNF423*, *CEBPA* and *PPARG*). Moreover; three of the four fibrogenic markers evaluated (*COL1A1*, *COL3A1* and *FN1*) and the number of muscle cells tended to be greater in male than in females foetuses. However, there were no effects of foetal sex on phenotypic indicators of adipogenesis and fibrogenesis. Collectively, these findings lead to the suggestion that the development of skeletal muscle is greater in males than in females during the intra-uterine life. Extensive efforts have been made by scientists to describe the sexual dimorphism of the evolutionary adaptation and allocation of maternal resources in mammals (Clark, 1978; Hinde, Carpenter, Clay, & Bradford, 2014; Smith, 1980; Trivers & Willard, 1973). Males seem to have a faster development of skeletal muscle during the foetal phase, while females have a greater intestinal development during the same period (Gionbelli et al., 2014). However, the causes and effects related to these findings are still not well elucidated.

In general, changes in maternal nutrition promote alteration in expression of myogenic and adipogenic markers at midgestation (greater in ON than in CO) but compensatory growth of foetuses from CO group cows demonstrated that the differences on expression of the markers mentioned above disappeared by late gestation. Previous study (Underwood et al., 2010) has reported that an increase in the quality of maternal diet for 60 days during the midgestation increased meat tenderness, adipose tissue and growth in the offspring; however, our results suggest that these potential effects does not occur when cows are offered a higher plain of nutrition over the maintenance requirements. Moreover, it should be noted that a potential increase in adipogenesis of skeletal muscle has been shown in foetuses from overnourished beef cows (Duarte et al., 2014). Our results revealed that maternal overnutrition can alter the trajectory and speed of skeletal muscle development (Tables 2, 3 and 4); however, the effects that remains until the late gestation are marginal. Therefore, by providing a higher nutritional level to pregnant cows changes occur in the trajectory of the development of skeletal muscle. However, it does not appear to change the potential of post-natal growth of muscle mass of the offspring due to the fact that by late gestation the results are not different in response to the different maternal nutritional treatments. Our findings about the differences between males and females during the intra-uterine muscular development phase suggest that studies on skeletal muscle development of bovine foetuses need to take into consideration foetal sex as an effect or use foetuses of the same gender.

In summary, our findings suggest that although maternal overnourishment causes increase in gene expression of several myogenic and adipogenic markers during midgestation it seems that a compensatory growth of foetuses from cows in the CO group occurred and skeletal muscle development did not differ at late gestation. These data suggest that providing a higher nutritional level for pregnant cows changes the trajectory of the skeletal muscle development but this potential is not continuous through the late stage of gestation and the outcome of foetal skeletal muscle development of dual purpose bovines may not be affected. However, further investigations need to take place to determine whether having a higher rate of development of skeletal muscle during midgestation will promote any benefit to those animals during a post-natal period. Moreover, our data shows an evidence of sexual dimorphism in skeletal muscle development during foetal stage.

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CONFLICT OF INTEREST

No competing financial, personal or professional interests have influenced writing of this paper. This manuscript has not been submitted anywhere else for possible publication.

AUTHORS' CONTRIBUTIONS

T.R.S.G., P.P.R., C.M.V., M.I.M., S.C.V.F. and M.P.G. designed research; T.R.S.G., P.P.R.C.S., B.C.C., C.S.C. and M.A.S.N. performed research; M.S.D. contributed new reagents/analytic tools; T.R.S.G. and M.P.G. analyzed data; and T.R.S.G., L.D.P, M.S.D. and M.P.G. wrote the paper.

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